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Ultrastructural changes in the digestive system of *Indoplanorbis exustus* (Gastropoda : Planorbidae) exposed to the molluscicides, niclosamide and *Brassaia actinophylla*

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Abstract

The adult snails, *Indoplanorbis exustus* were exposed to the synthetic molluscicide, niclosamide, and the crude - aqueous extract of *Brassaia actinophylla* at the LC50 concentrations (0.009 mg/l and 23.73 mg/l, respectively) for 24 hours. The results showed relatively similar ultrastructural changes in the digestive system of snails exposed to niclosamide and *B. actinophylla*. The general cytological alterations that occurred in the epithelia of esophagus, intestine and digestive gland were the formation of blebs, increased fusion of vacuoles, infolding and thickening of basal lamina, formation of myelin-like figures, degranulation and dilation of rough endoplasmic reticulum, swelling of mitochondria and cristae, and an increase in mucus production. Most of these reactions were more severe in snails treated with *B. actinophylla* than those exposed to niclosamide.

Key words : *Indoplanorbis exustus*, niclosamide, *Brassaia actinophylla*, digestive system

Introduction

There are a relatively few publications concerning ultrastructural changes of the digestive system in gastropods exposed to molluscicides (Adewunmi & Ogbe, 1986; Triebskorn, 1989; 1991; Triebskorn & Künast, 1990; Bourne *et al.*, 1991; Bode *et al.*, 1996). The most extensive studies have been done on slugs such as, *Deroceras reticulatum* (Muller) (Triebskorn, 1989; 1991; Triebskorn & Künast, 1990), where it was reported that molluscicides produced cytological changes in the general outline of cells and in their apical and basal surfaces. The columnar cells of the digestive tract were greatly elongated, and often gaps opened between the epithelial cells and the underlying muscle and nerve tissues (Triebskorn, 1989; Triebskorn & Künast, 1990). Changes at the cell apices included reduction of microvilli, formation of surface blebs and production of a hyaline surface coat overlying the microvilli or cilia (Triebskorn & Künast, 1990). The most striking changes in the basal surface of the cells were development of cell extensions, thickening of the basement membrane, and development of gaps between the epithelium and the basement membrane (Triebskorn, 1989; Triebskorn & Künast, 1990; Bourne *et al.*, 1991).

The most typical cytological changes in the cytoplasm were degranulation and dilation of rough endoplasmic reticulum, formation of membrane whorls, disorganization of Golgi cisternae, intensified fusion of vacuoles, swelling of mitochondria and reduction of cristae, and reduction of storage products (Triebskorn, 1989; 1991; Triebskorn & Künast, 1990). In the nucleus, karyolysis, reduction of heterochromatin, dilation of nuclear envelope and formation of crystalline inclusions were reported (Triebskorn & Künast, 1990).

Most of the ultrastructural studies on cytological changes of the digestive system as results of molluscicides have concentrated only on stylommatophoran slugs and one basommatophoran snail, *Biomphalaria glabrata* (Say) (Bode *et al.*, 1996). Thus, the present electron microscopic study was designed to investigate different cellular responses to a synthetic molluscicide (niclosamide) and a plant extract (*Brassaia actinophylla*) in the digestive system of the freshwater pulmonate, *Indoplanorbis exustus* (Deshayes), the snail intermediate host of *Schistosoma spindale* Montgomery.

Materials and methods

Laboratory-bred, *I. exustus*, with a shell diameter of 1.5-1.8 cm, were used. Batches of thirty snails each were exposed to niclosamide and crude - aqueous extract of *B. actinophylla* at the LC50 concentrations (0.009 mg/l and 23.73 mg/l, respectively) for 24 hours (Upatham *et al.*, in press). The control group consisting of another 30 snails, was subjected to aged tap water.

After the exposure, ten snails from each group were sacrificed. They were relaxed with menthol crystals for 30 minutes and their shells were removed. The organs of the digestive system, esophagus, stomach and intestine, were dissected out and prepared for transmission electron microscope (TEM), viz. first fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 hours, washed in the same buffer and postfixed in 1% OsO_4 in 0.1 M phosphate buffer for 2 hours. The specimens were dehydrated through a graded series of ethanol and embedded in Araldite 502 epoxy resin. Semithin sections were cut with glass knives and stained with methylene blue. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a Hitachi H300 transmission electron microscope operating at 75 KV.

Results

Esophagus

In control snails, the esophagus epithelium consists of two cell types, ciliated columnar cell (columnar storage cell) and mucus-secreting goblet cell (mucus cell) (Fig.1A). The columnar cells bear both microvilli and cilia on the apex (Fig.1B). They contain numerous mitochondria, numerous glycogen rosettes (Fig. 1B), regular membranes of rough endoplasmic reticulum (RER) and Golgi apparatus. The nuclei are rich in heterochromatin and occupy the middle to basal region of the cell. The mucus cells are characterized by conspicuous RER with spacious lumen, mucus vacuoles and glycogen rosettes (Fig.1C). The nuclei are located at the basal region. The infoldings of basal surfaces of both cell types are small, and the basement membrane is very thin (Fig.1D).

The columnar storage cells of both niclosamide and *B. actinophylla* intoxicated snails are still crowded with glycogen granules but the glycogen rosettes are no longer visible (Fig. 2A). There are degranulation and dilation of RER (Fig.2B). No conspicuous alteration was observed in the Golgi apparatus and mitochondria (Fig.2A).

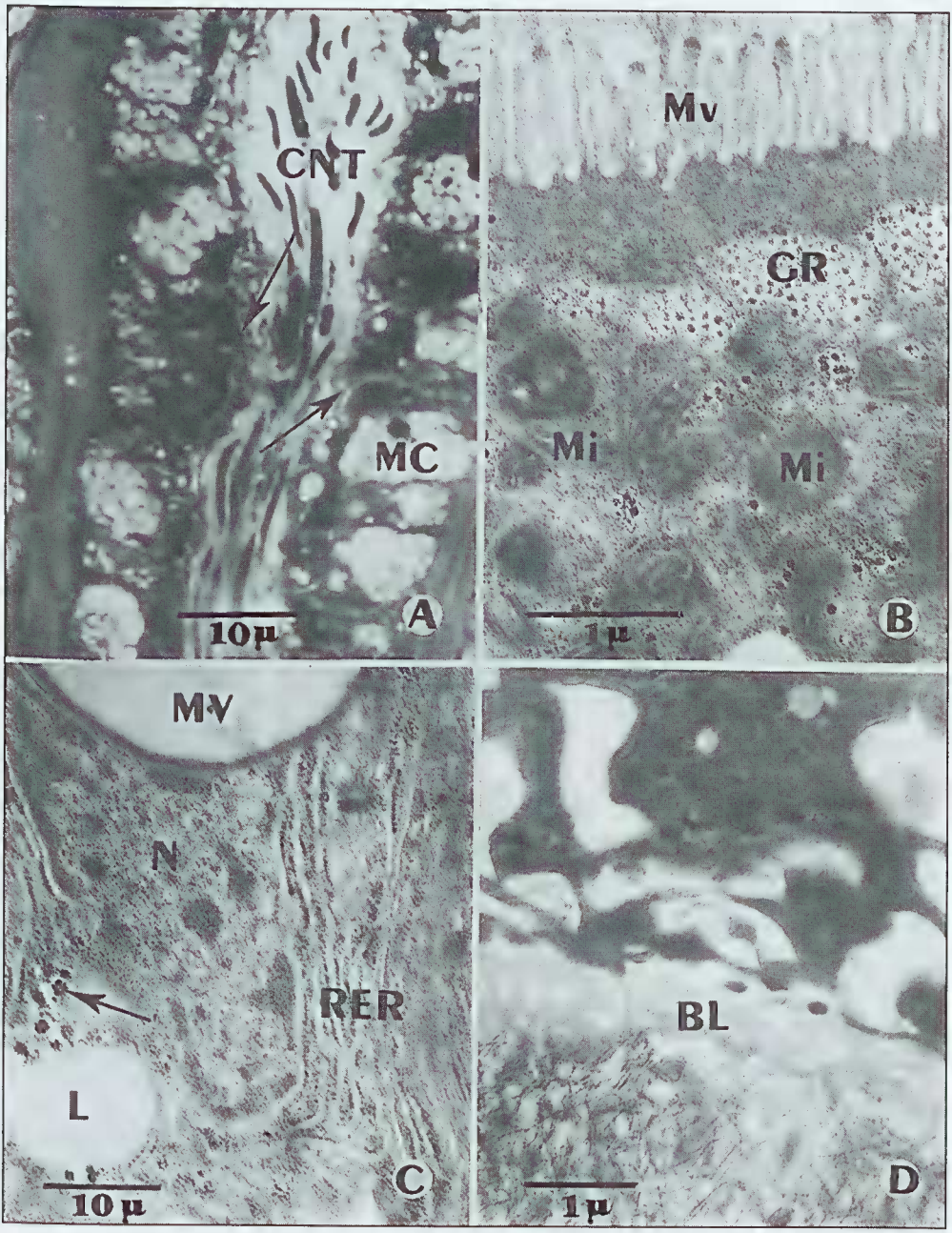

Figure 1. Light (**A**) and electron (**B-F**) micrographs of esophagus epithelia in control snails. **A.** Low magnification showing storage cell (arrows) and mucus cell (MC) located on connective tissue (CNT). **B.** Apex of storage cell bearing microvilli (Mv). Beneath the border of microvilli, mitochondria (Mi) and glycogen rosettes (GR) are visible. **C.** Mucus cell showing nucleus (N), lipid droplet (L) and mucus vacuole (MV), rough endoplasmic reticulum (RER) and glycogen rosettes (arrow). **D.** Basal part of epithelial cells with thin and smooth basal lamina (BL).

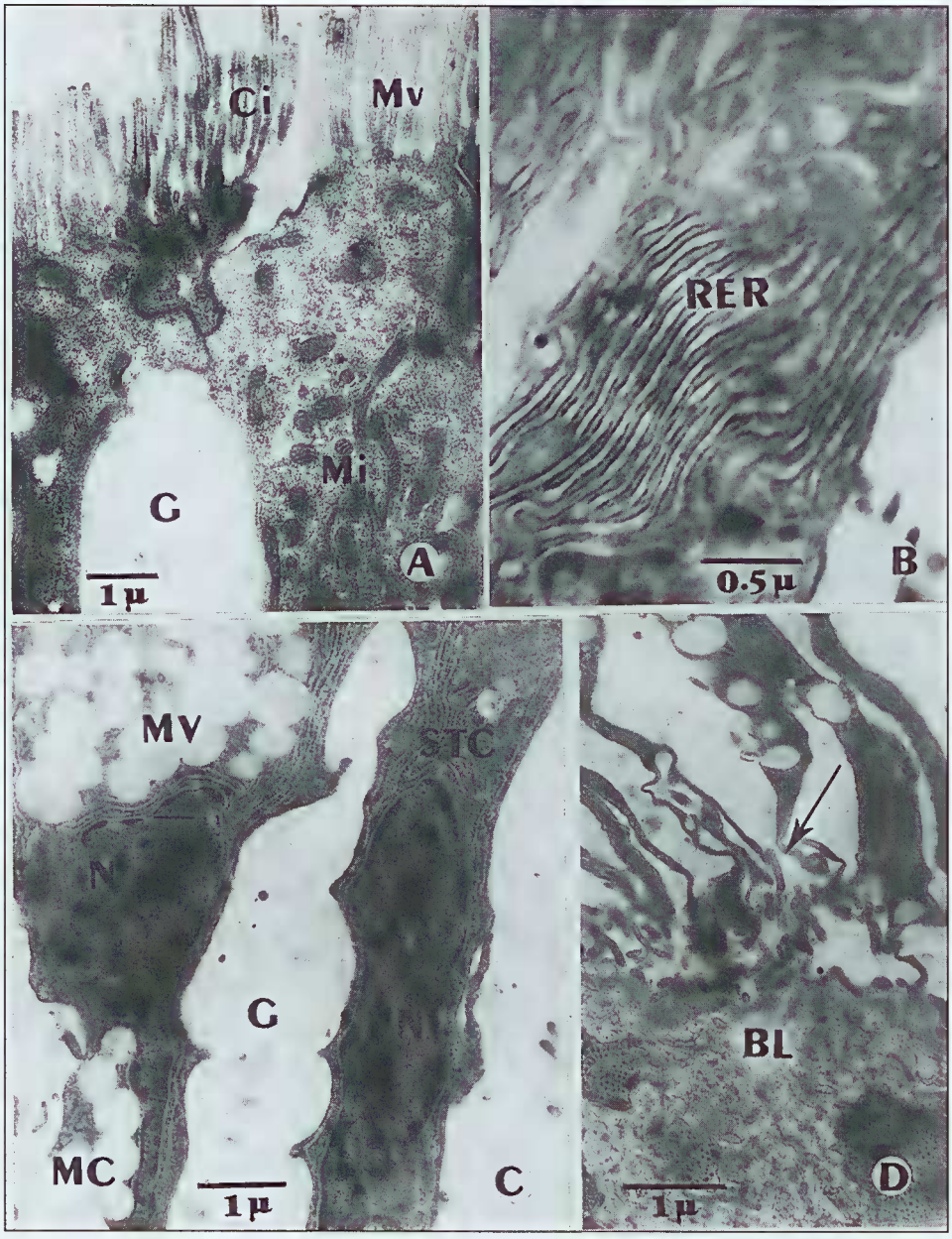

Figure 2. Electron micrographs of esophagus epithelia in snails exposed to niclosamide and *B. actiophylla.* **A.** Storage (STC) in *B. actinophylla* intoxicated snails. Storage cell contains numerous mitochondria (Mi). Note the presence of a large gap (G) between cells. Mv = microvilli, Ci = cilia. . **B.** Storage cell showing degranulation and slight dilation of rough endoplasmic reticulum (RER). **C.** High magnification showing nuclei (N) of mucus cell (MC) and storage cell (STC) with large patches of heterochromatin. Fusion of small vesicles to form large mucus vacuoles (MV) in mucus cell is observed. G = gap. **D.** Basal lamina (BL) showing thickening and increase in infoldings (arrow).

The most striking cytological alterations occur in the mucus cells. There is an increased production of mucus and an intensified fusion of mucus vacuoles (Fig.2C). In the snails intoxicated with either molluscicides, the columnar storage and mucus cells contained nuclei of irregular shape with patches of heterochromatin (Fig. 2C). Large gaps had formed between two adjacent columnar cells (Figs. 2A,2C). In addition, the number of infoldings of the basal lamina had increased (Fig.2D).

Intestine

In control snails, the intestinal epithelium is made up of three cell types, columnar storage cells, secretory cells and mucus cells (Figs. 3A, 3B). Secretory cells predominate.

The storage cells bear both microvilli and cilia and contain numerous mitochondria, regularly-arranged RER, Golgi apparatus and glycogen rosettes (Fig. 3C). Nuclei with nucleoli and patches of heterochromatin are located in the center or subcenter of the cells (Fig.3B). The secretory cells, the most numerous cell type, are characterized by the presence of numerous secretory granules, mitochondria, stacks of RER and well-developed Golgi apparatus (Figs.3B, 3D). The mucus cells are rarely observed and those present contain large mucus vacuoles (Fig3A). The basal part of the epithelium contains small infoldings and the basal lamina is thin (Fig.3E).

The cytological alterations of the intestinal epithelium in niclosamide and *B. actinophylla* intoxicated snails are similar (Fig.4). There is a decrease in number of secretory cells and an increase in number of mucus cells with large mucus vacuoles. Fusion of small vacuoles into large mucus vacuoles was observed (Fig.4A).

The columnar storage cells have undergone some striking changes. There is an increase in number of lysosomes (Fig.4B). Mitochondria remain numerous but show some swelling of cristae (Fig.4B). There is degranulation and dilation of RER (Fig.4C). Membrane whorls of RER could be observed (Fig.4D). Glycogen rosettes are not present. The cell membranes are destroyed in some areas and gaps occur between cells (Fig.4D). The alterations in the nucleus include lightening of karyoplasm and reduction of heterochromatin (Fig.4D). The basal lamina of epithelial cells has become thickened with the increase in infoldings (Fig.4E).

Digestive gland

Control snails

The digestive gland of *I. exustus* is arranged in tubules that are bound together by connective tissue. Four main types of cell can be distinguished in the digestive gland, digestive cell, secretory cell, excretory cell and thin cell (Fig.5A).

Digestive cell Digestive cells which form the major component in the gland, are columnar in shape, vary greatly in length within the same tubule and usually possess microvilli (Fig.5B). The conspicuous vacuoles vary in size (Fig.5B). A few mitochondria and scattered RER are present in the cytoplasm. Lipid and glycogen rosettes can be found. The nuclei of these cells are located in the basal region (Fig.5B).

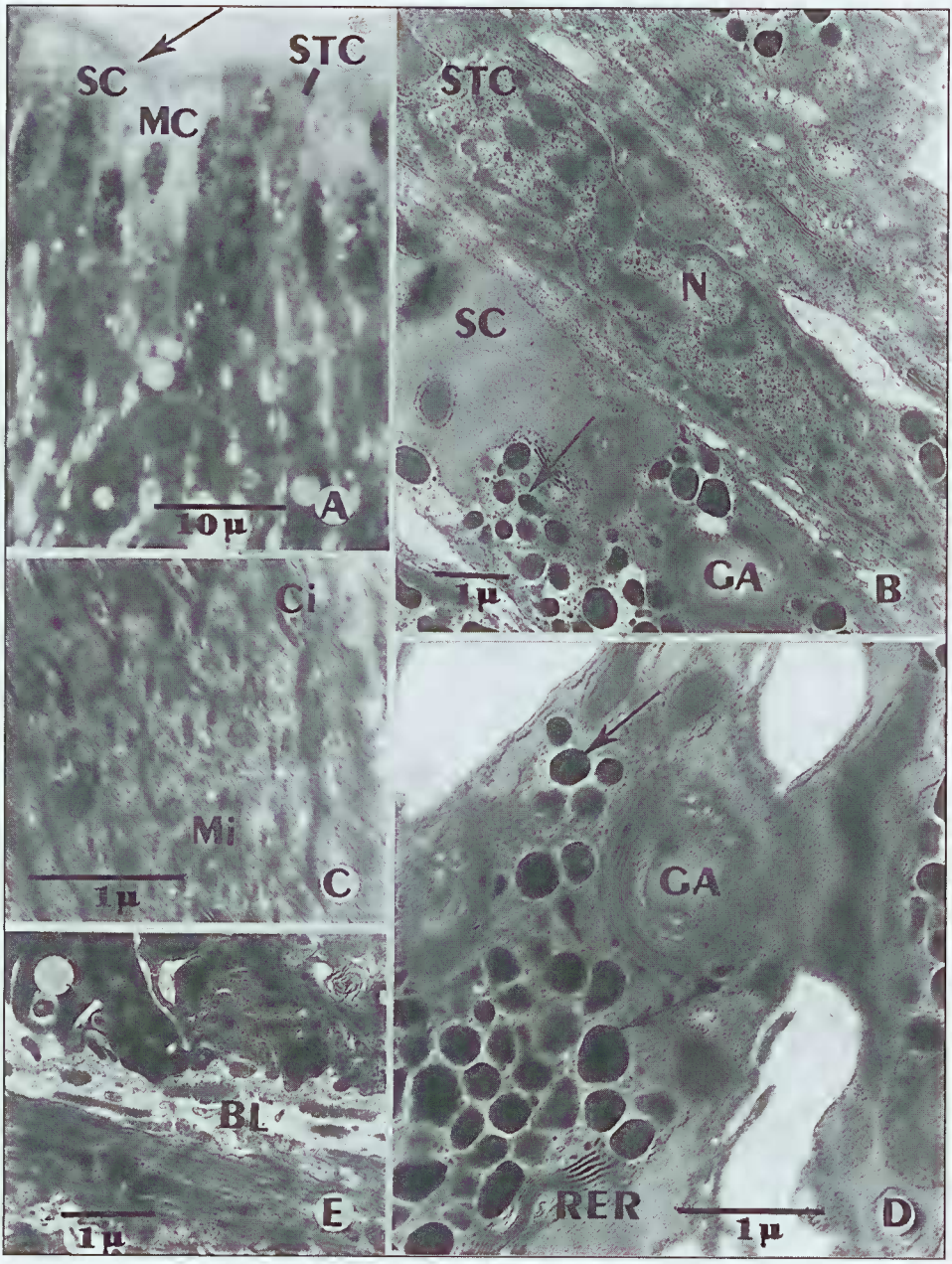

Figure 3. Light (**A**) and electron (**B-F**) micrographs of intestinal epithelia in control snails. **A.** Lining epithelium of intestine, showing storage cell (STC), secretory cell (SC) and mucus cell (MC) with brush border (arrow) composed of cilia and microvilli. **B.** Low magnification of storage cell (STC) with long nucleus (N) and secretory cell (SC) with secretory vesicles (arrow) and Golgi apparatus (GA). **C.** Apex of storage cell (STC) bearing cilia (Ci). Beneath the apical surface of the cell, mitochondria (Mi), are visible. **D.** Secretory cell showing Golgi apparatus (GA), and secretory vesicles with electron-dense material (arrows). RER = rough endoplasmic reticulum. **E.** Basal part of epithelial cells with thin and smooth basal lamina (BL).

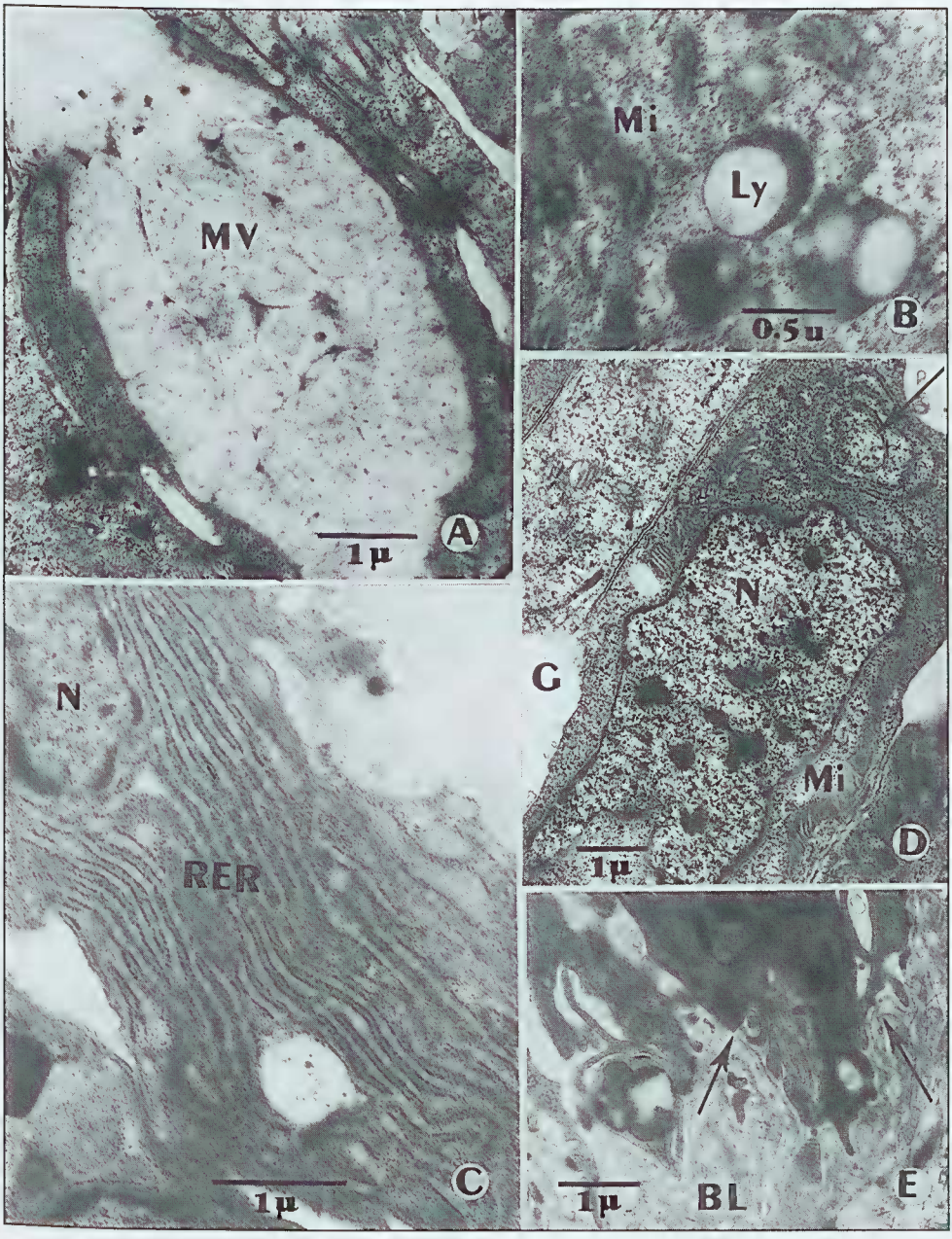

Figure 4. Electron micrographs of intestinal epithelia in niclosamide and *B. actinophylla* intoxicated snails. **A.** Mucus cell showing mucus vacuole (MV) with mucus which is being secreted into the lumen. **B.** Storage cell showing lysosomes (Ly) and mitochondria with swelling cristae (Mi). **D.** Storage cell showing membrane whorls of rough endoplasmic reticulum (RER) in cytoplasm and a decrease of heterochromatin in the nucleus (N). G = gap. Mi = mitochondria. **C.** Storage cell showing dilation and degranulation of rough endoplasmic reticulum (RER). N = nucleus. E. Basal lamina (BL) showing numerous infoldings (arrows).

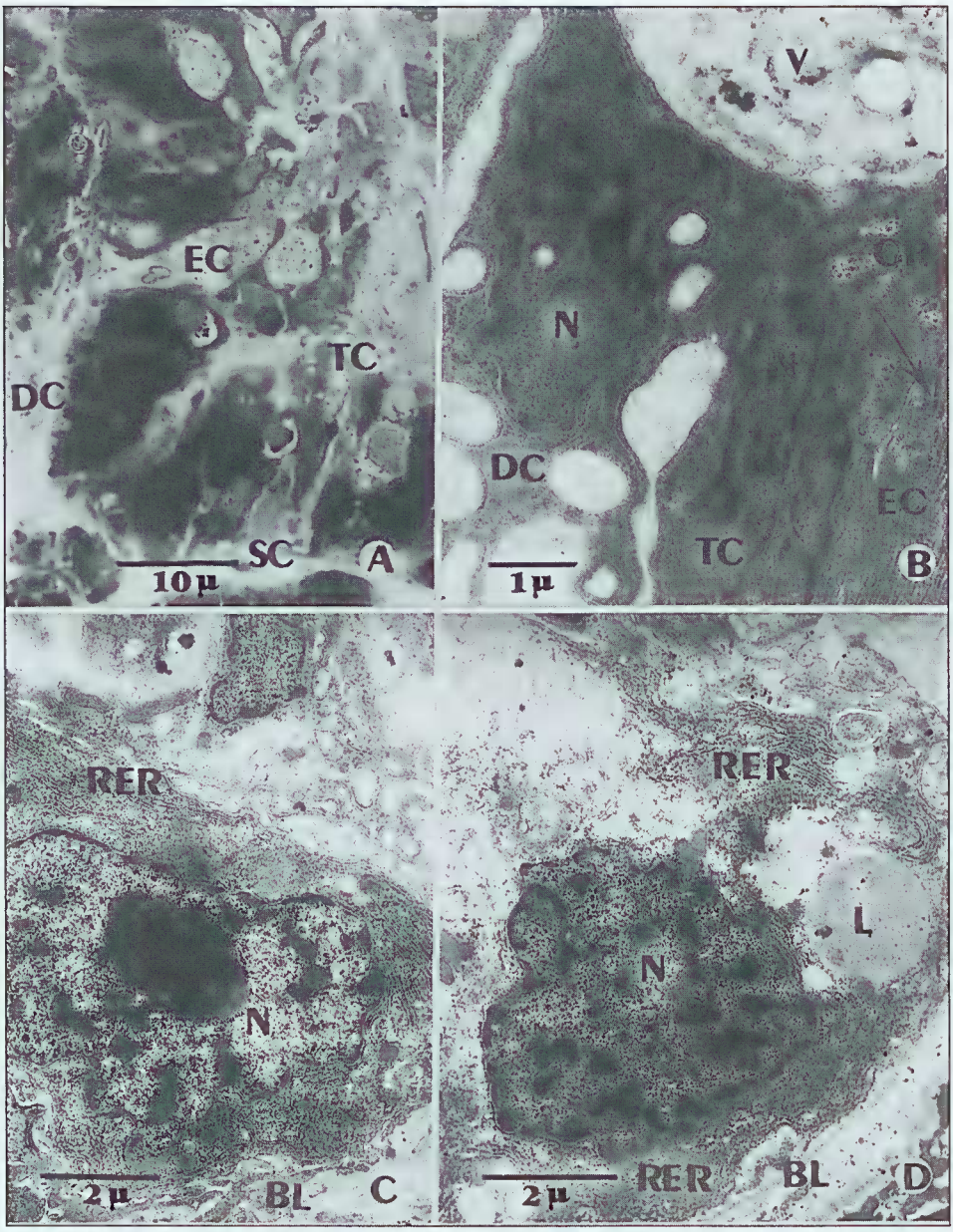

Figure 5. Light (**A**) and electron micrographs (**B-E**) of digestive gland epithelia in control snails. **A.** Epithelium of digestive gland tubule showing digestive cells (DC), excretory cells (EC), secretory cells (SC) and thin cells (TC). **B.** Various cell types of digestive gland, digestive cell (DC) with large vacuole (V) containing membranous material, thin cell (TC) with glycogen rosettes (GR), excretory cell (EC) with stacks of rough endoplasmic reticulum (arrow). N=nucleus. **C.** Secretory cell with abundant rough endoplasmic reticulum (RER). BL = basal lamina, N = nucleus. **D.** Excretory cell with abundant rough endoplasmic reticulum (RER). N = nucleus, L = lipid droplet, BL = basal lamina.

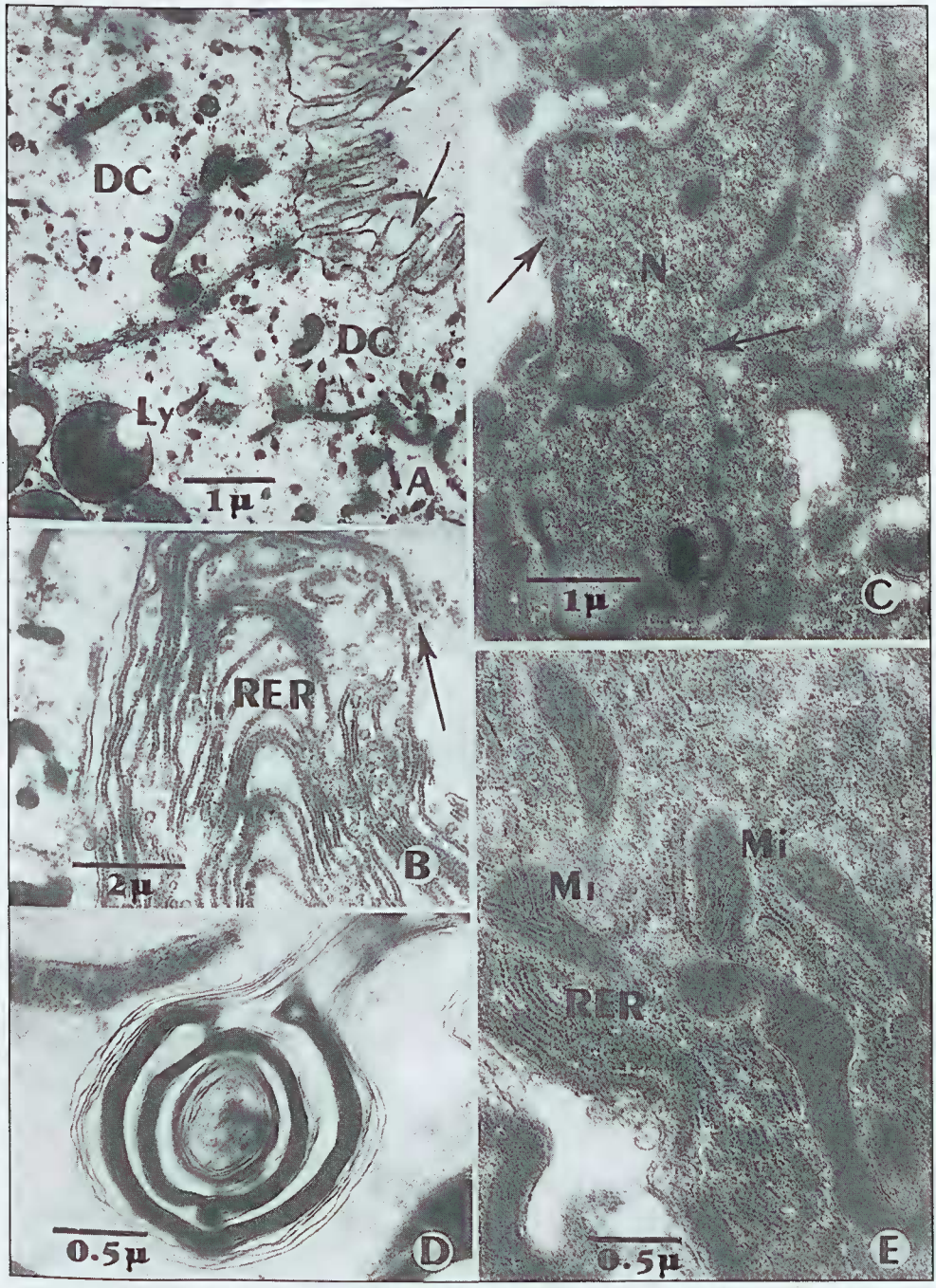

Figure 6. Electron micrographs of digestive gland epithelia in niclosamide intoxicated snails. **A.** Apical part of cells, showing surface blebs and dilation of microvilli (arrows) of digestive cells (DC). Ly = lysosome. **B.** Digestive cell showing fragmentation (arrow) of rough endoplasmic reticulum (RER). **C.** Digestive cell showing disruption of nuclear membrane (arrows). N = nucleus. **D.** Secretory cell showing myelin-like figure. **E.** Secretory cell showing irregular arrangement and swelling of cristae in mitochondria (Mi) and degranulation and fragmentation of rough endoplasmic reticulum (RER).

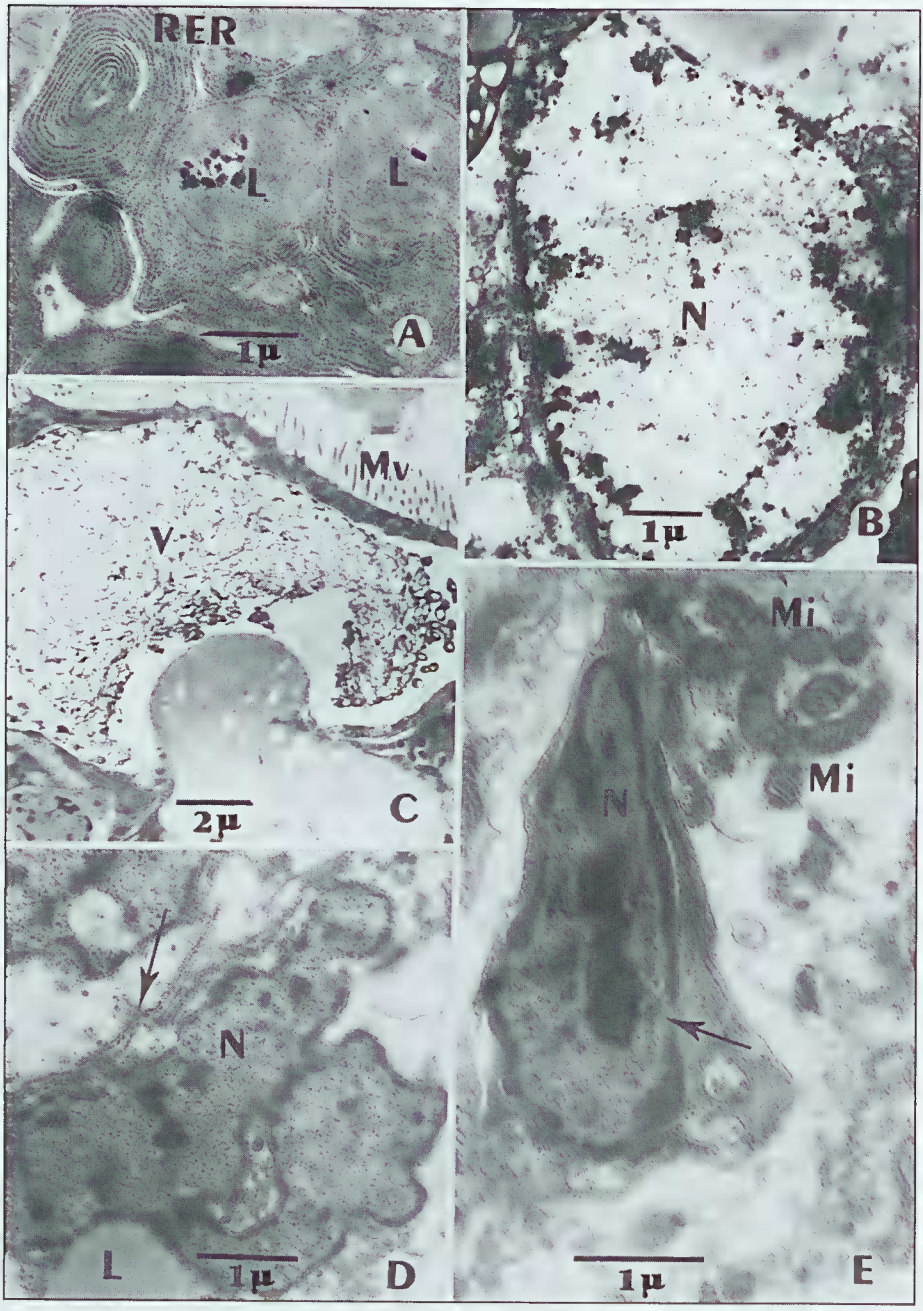

Figure 7. Electron micrographs of digestive gland epithelia in *B. actinophylla* intoxicated snails. **A.** Excretory cell showing membrane whorls of rough endoplasmic reticulum (RER) around lipid droplets (L). **B.** Excretory cell showing karyolysis of nucleus (N). **C.** Excretory cell showing large vacuole containing electron-dense material (V). Mv = microvilli. **D.** Secretory cell showing irregular nucleus (N) and fragmentation of rough endoplasmic reticulum (arrow). L = lipid droplet. **E.** Thin cell showing pycnotic nucleus (N) with disruption of nuclear membrane (arrow) and swelling of cristae and mitochondria (Mi).

Secretory cell Secretory cells usually occur singly in the corner of the tubules. They are pyramidal or conical in shape with broad bases, occasionally abutting into the hemolymph space (Fig.5C). There is a large central or basal nucleus with scattered patches of heterochromatin (Fig.5C).

Excretory cell Excretory cells are larger than digestive cells. The apical surface of this cell type has a dense brush border of microvilli. In the cytoplasm, a small Golgi apparatus, a small number of lipid droplets, mitochondria and extensive RER can be found (Fig.5D). The nucleus is usually positioned in the basal region (Fig.5D).

Thin cell Thin cells are usually situated between digestive cells and secretory cells. They have a border of microvilli. Several mitochondria, free ribosomes and glycogen rosettes are found in the apical cytoplasm (Fig.5B). The nucleus is situated in the center of the cell (Fig.5B).

Molluscicide intoxicated snails

Niclosamide and the crude - aqueous extract of *B. actinophylla* caused alterations in the general outline of cells and in their apical and basal surfaces. The cells of the digestive gland have become irregularly shaped and gaps developed between the epithelial cells. Cytoplasmic protrusions (small surface blebs) were formed. There was slight elongation of microvilli. Glycogen rosettes were no longer visible in the cytoplasm (Fig.6A).

Digestive cell The most striking cytological alterations in the digestive cells of snails exposed to molluscicides were fragmentation of membrane whorls of RER (Fig.6B), fusion of small vacuoles and a decrease in glycogen. In the nucleus, the nuclear membrane was partially destroyed and there was a reduction in heterochromatin (Fig.6C).

Secretory cell Cytological alterations in secretory cells were similar to those in digestive cells. There was a formation of myelin-like figures (Fig.6D). Swelling of mitochondria and cristae was observed (Fig.6E). The nucleus became irregular in shape with reduction in heterochromatin (Fig.7D).

Excretory cell Cytological alterations in excretory cells are quite similar to those in digestive and secretory cells. There are dilation and fragmentation of RER and swelling of mitochondria and cristae. In addition, there was a formation of membrane whorls of RER around the lipid droplets (Fig.7A). Alterations in the nucleus were disruption of nuclear membrane and karyolysis (Fig.7B). In the most severe cases, the cytoplasm was replaced by a large vacuole (Fig.7C) or the whole cell content was extruded into the lumen.

Thin cell Cytological alterations in thin cells were similar to those in secretory and excretory cells with a dilation of RER, swelling of mitochondria and cristae, pycnosis of nucleus and disruption of nuclear membrane (Fig.7E).

Discussion

The general cytological alterations that occurred in the epithelium of the digestive tract of *I. exustus* exposed to niclosamide and *B. actinophylla* were the formation of blebs and the increased fusion of vacuoles, an increase in infoldings of basal lamina, degranulation and dilation of RER, a formation of myelin-like figures, and an increase in mucus production. Most of the reactions were more

severe in snails intoxicated with *B. actinophylla* than those exposed to niclosamide.

The formation of blebs and the increased fusion of vacuoles as results of toxicants or molluscicides had been described in some molluscs. The study of Triebskorn & Künast (1990) on the digestive cells of digestive gland of *D. reticulatum* showed that after intoxication, the intensified fusion between small and large vacuoles resulted in an increase in the number of large vacuoles. In the present study, the increase of large vacuoles and increased fusion of small and large vacuoles occurred in both niclosamide and *B. actinophylla* intoxicated snails. These might result from the interaction of the lipophilic molluscicide with membranes which might induce changes in composition, fluidity and finally stability of the membranes (Triebskorn & Künast, 1990).

Another histological alteration that occurred in the epithelial lining of esophagus and intestine in snails exposed to the molluscicides was the increase in infoldings of basal lamina. Kruatrachue *et al.* (in press) also found the thickening of basement membrane in *Filopaludina (Siamopaludina) martensi martensi* (Frauenfeldt) exposed to lead at the LC80 concentration for 96 hours. This alteration also occurred in *D. reticulatum* exposed to carbamate molluscicide (Triebskorn & Künast, 1990). Triebskorn & Künast (1990) suggested that thickening of the basement membrane might prevent further penetration of the toxin from either the lumen of the digestive tract or from the hemolymph space.

Degranulation and dilation of RER and the formation of myelin-like figures were observed in storage cells of esophagus and intestine, and in digestive and excretory cells of digestive glands of intoxicated *I. exustus*. Reactions of the RER have often been seen in both vertebrates (Sivarajah *et al.*, 1978; Klaunig *et al.*, 1979) and invertebrates, such as mussels (Nott and Moore, 1987) and *D. reticulatum* exposed to molluscicides (Triebskorn 1989; 1991, Triebskorn & Künast, 1990). Several reactions in subcellular organelles such as degranulation of endoplasmic reticulum and the formation of membrane whorls of fingerprint-like structures have been considered as specific stress symptoms or lesions (Rez, 1986). They are general changes of the cell in response to toxicants (Rez, 1986). Most of these reactions are attributed to membrane destabilization and increased membrane permeability to ions under the influence of toxicants (Sparks, 1972).

Increases of mucus in mucus cells and also of mucus cell number were observed in the esophagus and intestine of intoxicated *I. exustus*. The increase of mucus in the lumen of the digestive tract and production of mucus vacuoles due to increased activity of the secretory system (endoplasmic reticulum and Golgi apparatus) are immediate responses to the ingestion of lethal doses of molluscicides. The mucus might serve to dilute toxin and protect the cells of the digestive tract from damage by molluscicides. These reactions of exudate mucus had been observed in *D. reticulatum* (Triebskorn & Künast, 1990). It has been proposed that mucus cells have a protective and detoxifying function (Zylstra, 1972 a; 1972b; 1972c; Moya & Rallo, 1975). Moreover, snails are capable both of increasing the quantity, and of varying the quality, of the chemical composition of mucus (Triebskorn & Ebert, 1989). The exudation of acidic mucus can be regarded as a kind of incidental detoxification, because the toxin is less stable under acidic conditions. Nevertheless, the reason for the alteration in the

chemical composition of the mucus is not known (Triebskorn & Künast, 1990). We agree with the conclusion of Triebskorn & Künast (1990) that the induction of mucus secretion could finally lead to the desiccation and death of snails. The intensified exudation of mucus can kill snails and specific molluscicides do not only enhance secretion of mucus but also damage the ultrastructure of cells. Airey *et al.* (1989) regarded the mucus cells as one of the target for specific molluscicidal interference.

An increased use of energy on resources to initiate protective or detoxification processes might lead to a quick reduction of lipid and glycogen in the storage cells. Triebskorn & Künast (1990) also described the reduction of glycogen and lipid in storage cells of *D. reticulatum* intoxicated by carbamate. Dillaman (1980) reported the disappearance of glycogen rosettes in the ureter cells of kidney in the freshwater snail, *Helisoma anceps* Menke exposed to low concentrations of cadmium. We also observed a reduction in glycogen content in the storage cells of esophagus and digestive cells of the digestive gland of *I. exustus* exposed to niclosamide and *B. actinophylla.*

The main cell types of digestive gland are digestive cell, secretory cell, thin cell and excretory cell. Similar cells have been described for *Agriolimax reticulatus* (Muller) (Walker, 1970). The secretory cells serve secretory functions of storage and detoxification and have a characteristic conical outer shape. The excretory cells are characterized by diversity of vacuoles with electron-dense material which is excreted into the lumen of the gland. The digestive cells digest the food by pinocytosis (Bode *et al.*, 1996). In the snails *I. exustus* intoxicated with molluscicides, the digestive gland was highly affected. Some tubules were observed to be in the process of autolysis. The increased amount of lysosomal activity and extensive autolysis were also reported in the hepatopancreas of fiddler crab, *Uca minax* exposed to naphthalene (Robinson & Dillaman, 1985). In pathological conditions, autolysis of cells is commonly the result of the release of digestive enzymes into the cytoplasm after destruction of the lysosomal membranes.

Similar cytological alterations were observed in different cell types of digestive gland (digestive, excretory, secretory and thin cells) of intoxicated *I. exustus.* There was dilation and fragmentation of RER, swelling of mitochondria and cristae, pycnosis and karyolysis of nucleus and disruption of nuclear membrane. These ultrastructural changes are usually observed in invertebrates exposed to sublethal concentrations of pollutants (Papathanassiou & King, 1983; Robinson & Dillaman, 1985; Marigómez *et al.*, 1990). Dilation of rough endoplasmic reticulum and nuclear pycnosis were observed in the kidney epithelial cells of *Littorina littorea* (Linnaeus) treated with sublethal concentration of cadmium (Marigómez *et al.*, 1990) and in the hepatopancreas of *U. minax* exposed to naphthalene (Robinson & Dillaman, 1985). Although the rough endoplasmic reticulum was not destroyed, its structure was altered to the extent that its synthetic function could have been impaired (Robinson & Dillaman, 1985).Nuclear pycnosis as observed in the digestive gland cells of *I. exustus* and in the kidney epithelial cells of *L. littorea* usually indicates a serious injury to metabolic or regulatory processes.

The reactions of mitochondria, swelling and reduction of cristae and disruption of mitochondrial membrane are interpreted as a sublethally induced stress response to molluscicides or pollutants. Papathanassiou & King ((1983) reported irregular mitochondria with reduction of cristae in the gill lamellae of common shrimp, *Palaemon serratus* exposed to cadmium. Disruption of mitochondrial membrane was reported in the renal epithelium of *L. littorea* treated with cadmium (Marigómez *et al.*, 1990), in the absorptive cells of hepatopancreas in *U. minax* exposed to naphthalene, and in the gill lamellae of *P. serratus* exposed to cadmium. These alterations of mitochondria could reflect a response to factors inhibiting the passage of substrates for oxidation into the mitochondria leading to ATP-deficiency and subsequently metabolic activity (Papathanassiou & King, 1983; Robinson & Dillaman, 1985). Triebskorn & Künast (1990) assumed that the swelling of mitochondria and reduction of cristae could be induced in various ways by exogenous or endogenous stress. Even if the symptoms are similar, the causes of the response might be totally different.

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